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Comparison of the dietary bioavailability of copper sulphate and copper oxide nanomaterials in ex vivo gut sacs of rainbow trout: effects of low pH and amino acids in the lumen

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Comparison of the dietary bioavailability of copper sulphate and copper oxide nanomaterials in *ex vivo* gut sacs of rainbow trout: effects of low pH and amino acids in the lumen

David Boyle, Nathaniel J. Clark, Tarryn L. Botha and Richard D. Handy*

Diet is an important exposure pathway of engineered nanomaterials (ENMs) in fish, but the long duration of *in vivo* toxicity tests is a barrier to hazard assessment.

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PAPER

Comparison of the dietary bioavailability of copper sulphate and copper oxide nanomaterials in *ex vivo* gut sacs of rainbow trout: effects of low pH and amino acids in the lumen†David Boyle,^a Nathaniel J. Clark,^a Tarryn L. Botha^b and Richard D. Handy^{a,c}

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Diet is an important exposure pathway of engineered nanomaterials (ENMs) in fish, but the long duration of *in vivo* toxicity tests is a barrier to hazard assessment. This study used *ex vivo* gut sacs from rainbow trout (*Oncorhynchus mykiss*) to compare the bioaccumulation of Cu from CuO ENMs with CuSO₄ in the tissue layers of the gut after short-term (4 h) exposure. The effect of gut lumen conditions such as pH and the presence of amino acids on the behaviour and dissolution of the CuO ENMs was explored and how amino acids (cysteine, histidine) altered bioaccumulation in the gut sacs. Exposure to either CuSO₄ or CuO ENMs at pH 7.8 resulted in Cu accumulation in the mucosa of gut sacs prepared from the stomach, anterior-, mid- and posterior-regions of the gut when compared to saline only controls. In contrast, only CuSO₄ accumulated in the underlying muscularis which suggests transepithelial transport of CuO ENMs may be limited. Dialysis experiments showed that at pH 2, typical of the stomach, more than 90% of the Cu from the CuO ENMs became dissolved suggesting that the particulate is transformed to dissolved metal for most of the gastrointestinal tract. Amino acids greatly increased ENM dissolution, and in the gut sacs, L-cysteine caused elevated Cu accumulation in the muscularis after exposure to CuO ENMs at pH 7.8. In conclusion, CuO ENMs have lower bioavailability than CuSO₄ in gut sacs, but dissolution of ENMs in the gut can lead to dissolved Cu accumulation in fish.

Environmental significance

The diet is an important exposure pathway of engineered nanomaterials in aquatic animals and bioaccumulation is a key concern for hazard assessment of nanomaterials. This study used an *ex vivo* gut sac technique with rainbow trout to investigate the bioavailability of copper oxide nanomaterials at the gut epithelia in a fish. *In chemico* analyses of the transformations of nanomaterials under simulated gut conditions *i.e.* at acid pH and in the presence of amino acids, were also performed. The data show that copper oxide nanomaterials have lower bioavailability than copper sulphate but acid digestion and amino acids in the gut lumen will increase nanomaterial dissolution during gut transit and bioavailability at the gut epithelia.

1. Introduction

Persistence, bioaccumulation and toxicity (PBT) are key triggers for environmental risk assessment. Of these, the persistence in the environment (*e.g.* review by Lead *et al.*¹), and the ecotoxicity of engineered nanomaterials (ENMs) have been studied (*e.g.*, review by Schultz *et al.*²). However, bioaccumulation potential is less understood. In Europe, the Or-

ganisation for Economic Cooperation and Development (OECD) test guideline (TG) 305 which measures bioaccumulation potential in fish³ is often required for new substances, including ENMs.⁴ The *in vivo* determination of bioaccumulation was originally devised with reasonably soluble chemicals in mind,⁵ and it has proven challenging to maintain the exposure in the water column for ENMs in aquatic testing.^{6,7} Fortunately, the OECD TG 305 gives the option to conduct a dietary exposure method for chemicals that are difficult to handle in water; and this dietary method is also preferred for ENMs.⁴

However, the growing diversity of ENMs that are incorporated into industrial processes and consumer products presents a logistical challenge to environmental effects testing, especially if each form of ENM is considered as a 'new substance'.⁸ The size, shape, core of the material, and any

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surface functionalisation of ENMs are expected to alter their biological activity.^{9,10} To rationalise the volume of work, a tiered approach to bioaccumulation testing has been proposed,⁴ where 'ENMs of concern' can be quickly identified using alternative methods such as *in chemico* and *in vitro* techniques; and also reduce the burden of *in vivo* animal testing from an animal welfare perspective.

The isolated *ex vivo* gut sac technique is a well characterised tool originally used in physiology to investigate water and solute movements across the gut.¹¹ Once removed from fish, the tissues of the gastrointestinal tract remain viable for at least 4 h and exhibit stable membrane transport functions if bathed in an appropriately gassed physiological medium.^{12,13} This permits careful manipulation of the lumen environment without altering organ structure or function (*i.e.* the technique is *ex vivo*), and, once sutured tightly, measurement of the apparent net accumulation of the test substance on/into the different anatomical regions of the gut. This approach has been effective in demonstrating the bioaccumulation of dissolved metals in fish [*e.g.*, Hg in rainbow trout (*Oncorhynchus mykiss*),¹⁴ Ag in European flounder (*Platichthys flesus*),¹⁵] and it has also been recently used with ENMs in trout (TiO₂,¹⁶ Ag,¹⁷), although not yet for Cu-containing ENMs.

Copper (Cu) is an essential micronutrient that serves as a cofactor for a number of key proteins that are vital for life, *e.g.*, cytochrome c oxidase.¹⁸ In freshwater fish, Cu acquisition is predominantly from the diet.¹⁹ Measurements using ⁶⁴Cu in the gut sac technique have demonstrated that the intestinal uptake pathway(s) is saturable and therefore carrier mediated.¹³ Passive apical import of Cu into enterocytes is likely *via* the Cu-specific Ctr1 channel,^{13,20} divalent transporter, Dmt1,²¹ and an amino-acid stimulated Cu uptake pathway as evidenced for L-histidine.^{13,22} The rate limiting step for dissolved Cu uptake across the fish intestine, is the active export of Cu from the gut epithelial cells into the blood *via* the vesicular Cu-ATPase and chloride-dependent efflux pathways.¹² A similar level of understanding of the gastrointestinal uptake mechanisms for ENMs is lacking. The nanometre size of ENMs will prevent their (intact) uptake on solute transporters,²³ but instead, ENMs are likely absorbed into the gut epithelium of fish by endocytosis-related pathways. For example, Al-Jubory and Handy¹⁶ demonstrated uptake of TiO₂ ENMs by a nystatin-sensitive (endocytosis) pathway in perfused intestines of rainbow trout.

For a tiered approach to testing, such *ex vivo* evidence of uptake might justify moving to *in vivo* bioaccumulation tests for a given ENM.⁴ Although fish will ingest food contaminated with ENMs (TiO₂,²⁴ ZnO,²⁵), there are very few reports of dietary bioaccumulation of Cu from Cu ENM exposures in fish. Lammel *et al.*²⁶ reported negligible trophic transfer of Cu from sediment to worm to fish in an aquatic food chain. In contrast, at least two nutrition trials have found comparable Cu body burdens in fish fed equal concentrations of Cu as CuSO₄ or Cu-containing nanomaterials [red sea bream (*Pagrus major*);²⁷ Russian sturgeon (*Acipenser gueldenstaedtii*)²⁸]. However for Cu ENMs, the interpretation of such *in vivo* experi-

ments needs to be made with caution. For example in waterborne exposures, an acidic pH enhanced the apparent accumulation of Cu from Cu ENM in trout *in vivo*. This result was best explained by the low pH causing metal dissolution from the particles to increase bioavailability.²⁹ Similar concerns would apply to the acidic pH in the stomach of carnivorous fishes. Moreover, the amino acids histidine, cysteine and methionine (all present in fish food) are known Cu chelators³⁰ and are expected to modulate dietary Cu accumulation. An L-histidine dependent pathway for dissolved Cu uptake has been identified in trout,²² but the effects of amino acids on the bioavailability on Cu-based ENMs is unexplored.

This present study aimed to further demonstrate the utility of the gut sac preparation as a useful tool that could be used as part of a tiered approach to bioaccumulation testing. The specific objectives included measuring net Cu accumulation from CuO ENM exposures by the different anatomical regions of the gut; but also to conduct simulated gut condition experiments at acidic luminal pH values to explore any role of Cu dissolution of CuO ENMs, and separate experiments in the presence of histidine or cysteine to determine any amino-acid dependent modulation of Cu accumulation from CuO ENMs.

2. Methods

2.1 Rainbow trout

Rainbow trout of approximately 200 g were obtained from a commercial fish farm (Exmoor Fisheries, Somerset, UK). Following a period of 10 days quarantine to monitor the health of fish required prior to their acceptance into the recirculating fish facility systems and before approval for use in experiments, were maintained in dechlorinated conditioned re-circulating Plymouth tap water [(in mM, means ± SD, *n* = 3): Ca²⁺, 0.41 ± 0.01; K⁺, 0.04 ± 0.01; Mg²⁺, 0.09 ± 0.01; Na⁺, 0.43 ± 0.01] at 16 ± 1 °C and under a photoperiod of 12 h light and 12 h dark for approximately a week until used in experiments. Fish were fed trout chow, daily, until 48 h prior to experimentation when feed was withdrawn to allow evacuation of the gut (see below). All procedures with live fish were conducted in accordance with ethical approvals from the Home Office, UK, under the animals (scientific procedures) Act 1986 and in compliance with the EU directive 2010/63/EU.

2.2 Characterisation of behaviour of CuO ENMs under simulated gut conditions

Copper oxide ENMs were provided in dry powder form by PlasmaChem GmbH (Berlin, Germany), a partner in the Sustainable Nanotechnologies Project (SUN) which was funded by the EU 7th Framework Programme. The characterisation of the same batch of these ENMs has been reported elsewhere.^{31,32} The primary particle sizes of these uncoated CuO ENMs were (mean ± SD, *n* = 100): 18.2 ± 5.6 nm.³² Stock suspensions of CuO ENMs were prepared first in ultrapure H₂O (18.2 MΩ, ELGA, UK) and dispersed with sonication for 1 h (50/60 Hz, 35 Watts, FB15048, Fisherbrand) immediately prior to dilution to the required concentrations in synthetic

luminal saline (see details of composition below) for use in experiments. Stocks of dissolved Cu (as CuSO_4) were similarly prepared in ultrapure water but were not sonicated and diluted in saline as required. All suspensions were prepared on the day of use to avoid extended periods of ENM dissolution.

The agglomeration behaviours of the CuO ENMs in saline was investigated using nanoparticle tracking analysis (NTA, NanoSight, Malvern Instruments, Malvern, UK). Stocks of the ENMs were prepared in ultrapure water as described above and were then further diluted to 6.354 mg L^{-1} Cu (as CuO ENMs, see section 2.3) in either ultrapure water or saline. After dilution in water or saline the samples ($n = 3$) were analysed within 2 minutes. Analyses were not performed for CuSO_4 .

The rate of dissolution of CuO ENMs was measured by dialysis and according to the method in Al-Bairuty *et al.*²⁹ This was performed for separate samples in several media to simulate the passage of the ENMs through the changeable environment of the gastrointestinal tract. The synthetic luminal saline used [in mM: 117.5 NaCl; 5.7 KCl; 25.0 NaHCO_3 ; 1.2 NaH_2PO_4 ; 2.5 CaCl_2 ; 1.0 MgSO_4 ; 23.0 mannitol; 5.0 glucose; pH 7.8 (ref. 12)] was chosen to reflect the alkaline pH and concentrations of bicarbonate in the post-stomach environment of the rainbow trout intestine.³³ Dissolution of the CuO ENMs was also performed in luminal saline acidified with HCl to pH 2 and pH 4 to span the pH range measured in stomach of trout during acid digestion of feed.³³ Levels of dietary histidine inclusion in commercial fish diets may exceed 1% of the feed on a mass basis,³⁴ and given the importance of amino acids in Cu bioavailability, separate dialysis experiments were performed with the saline supplemented with 5 mM L-histidine or 5 mM L-cysteine (both Sigma-Aldrich, UK; purity $\geq 97\%$) at pH 7.8 to simulate the effects of amino acids in chyme on Cu dissolution.

Dialysis tubing (15 cm lengths, 12 kDa cut-off, Sigma, UK, $n = 3$ per treatment) was prepared according to the manufacturer's instructions, and tied closed at one end. Then, 3 mL of a stock of CuO ENMs at 0.3977 g L^{-1} in ultrapure water was pipetted into each dialysis bag. The open end of the tubing was then tied to close it and the tubing immersed in 297 mL of the appropriate saline (see above) in an acid-washed beaker containing a magnetic stirrer. Beakers were placed on to a stirrer plate and the contents of the beakers were gently stirred over 4 h. At 0 (before adding the dialysis tubing), 0.25, 0.5, 1, 2 and 4 h, samples (1 mL) were taken from outside the dialysis bags for total Cu measurements (see section 2.4). The saline was adjusted to the desired pH at 0 h with 1 N NaOH or concentrated HCl and was monitored throughout the 4 h incubation, and adjusted as necessary. The salines at pH 2 and pH 7.8 were stable throughout the experiment but the saline at pH 4 had risen to approximately pH 4.5 at 1 h; this was adjusted to pH 4 by the addition of HCl and was then stable for the rest of the experiment. Several additional controls were included in the experimental runs to confirm the observations made. To account for the potential adsorption of dissolved Cu to the beakers and dialysis tubing (especially at pH 7.8), CuSO_4 at $100 \mu\text{g Cu L}^{-1}$ was spiked into additional

beakers ($n = 3$) containing saline and 15 cm lengths of dialysis tubing (as above), and Cu concentrations measured in 1 mL samples throughout the 4 h incubation (see section 2.4). This concentration of Cu was selected based on previous measurements of Cu dissolution from these CuO ENMs in freshwater at pH 7.³²

It was also expected that any high rates of dissolution observed in the dialysis experiments would result in a reduction of particle size. To measure primary particle diameters, CuO ENMs were incubated in the saline at pH 4 and pH 7.8 ($n = 3$) for 4 h in identical conditions to those above for the dialysis experiments and then examined by transmission electron microscopy. Briefly, samples of the particles from the dialysis bags were collected on to TEM copper grids. The primary particle diameters of CuO ENMs ($n = 100$ ENMs per replicate, $n = 3$ independent replicates at pH 4 and pH 7.8) were then measured using ImageJ software³⁵ from images captured using a JEOL JEM 1400 transmission electron microscope (JEOL, South Korea).

2.3 Rainbow trout gut sacs

Rainbow trout gut sacs were prepared according to Clark *et al.*¹⁷ with minor modification for Cu. Trout were unfed for 48 h to facilitate clearing the gut lumen of food and then were humanely sacrificed with a strike to the cranium to stun and then immediately pithed (schedule 1 method in compliance with ethical approval), and then the entire gastrointestinal tract was carefully removed. The liver was then carefully tied off with sutures and discarded. The lumen of the gastrointestinal tract was then gently washed through with saline (recipe in section 2.2) at 15°C . Uptake of Cu (as CuSO_4 and CuO ENMs) was measured in four discrete gut compartments (stomach, anterior including caecae, mid and posterior intestine) that were easily separated according to morphology with a scalpel blade. One end of each tissue compartment was then closed with suture and the gut sacs were weighed empty. Gut sacs have been shown to remain viable for at least 4 h after removal from the fish^{12,13} and this duration of exposure gave the best opportunity to detect differences in Cu burdens between the treatment groups.

The first series of experiments explored apparent Cu accumulation in a normal gut saline at pH 7.8. Gut sacs ($n = 6$ fish per treatment) formed from all gut regions were filled to uniform fullness *via* PE50 tubing with saline (pH 7.8) containing 6.354 mg L^{-1} Cu (as CuSO_4 or CuO ENMs, equivalent to $100 \mu\text{M}$ dissolved Cu and prepared as described in section 2.2). Measured (ICP-MS) concentrations of Cu in saline stocks were within 90–105% of nominal values for all treatments. Gut sacs were then sutured closed, re-weighed to obtain the weight (volume) of suspension added, and then bathed in individual tubes with a small volume (20 mL stomach and anterior intestine, 5 mL mid and posterior-intestines) of the same physiological saline (serosal saline; no added test substance). Tubes were maintained at $15 \pm 1^\circ\text{C}$ (to match the aquarium conditions of the trout) in a water

bath and the saline (serosal side) was continuously gassed with 99.7% O₂:0.3% CO₂ for 4 h.¹⁶ The serosal saline was gassed to mimic the *in vivo* P_{CO₂} levels in trout. Control gut sacs were filled with saline, only, and were included in the experimental design to account for background concentrations of Cu in tissues. Subsequent experiments to investigate the effects of 5 mM L-histidine or 5 mM L-cysteine on the uptake of CuSO₄ and CuO ENMs (Cu concentration as above) focussed on mid and posterior-intestines, only (*n* = 5, cysteine; *n* = 6, histidine). The procedures used and samples gathered (described below) were the same for all experiments.

After 4 h incubation, the gut sacs were removed from tubes, dabbed dry on tissue paper to remove residual saline, and weighed again. Each sac was then carefully opened and the contents of the lumen were rinsed away with 5 mL saline

Net water flux (μL g⁻¹ h⁻¹, eqn (1)) was assessed gravimetrically for each gut sac from the change in volume (Δ*V*) over time (*T*) as follows (eqn (1)):

$$\text{Fluid flux} = \frac{\Delta V}{\text{tissue dwt} \times T} \quad (1)$$

The percentages (%) of 'new' Cu in the tissues and serosal salines after 4 h was calculated from the absolute amount of Cu in the tissue [*i.e.*, the Cu concentration measured in the tissue (μg g⁻¹ dwt), after correcting for the background Cu concentration in controls, multiplied by the dry weight (g)] and the absolute amount of Cu in the initial dose added to the gut lumen (μg, calculated from the volume of Cu stock injected into the gut sacs) using (eqn (2)):

$$\% \text{new Cu accumulation} = \frac{([\text{Cu}]_{\text{tissue compartment}} - [\text{Cu}]_{\text{background}}) \times \text{tissue dwt}}{\text{Absolute dose of Cu injected into gut lumen}} \times 100 \quad (2)$$

(hereafter called the 'luminal rinse'¹⁷) and then loosely bound surface Cu was removed in a second wash (5 mL) containing the metal ion chelator EDTA (1 mM, hereafter called the 'EDTA rinse'). EDTA is a divalent metal ion chelator and will bind any Cu²⁺ and the Mg²⁺/Ca²⁺ in the rinse. The latter effect on divalent ions will tend to reduce aggregation according to Derjaguin–Landau–Verwey–Overbeek (DLVO) theory (thus making the particles more labile to wash off), but mainly will alter the viscous properties of the surface mucus by removing cations³⁶ so that any loose surface material (mucus, particles, other debris) is more easily removed from the gut surface. After very gently dabbing the lumen side of the tissue with tissue paper to carefully remove excess moisture, the mucosal layer was then collected by scraping with a glass slide and separating it from the underlying muscularis.

Total Cu concentrations were measured in the mucosal and muscle layers of the tissue, in the luminal rinse and EDTA rinse, and in a sub-sample of the serosal saline at the end of each experiment. For the gut sacs formed from the anterior intestine, the caecae were removed and Cu concentrations were analysed separately from the mucosal and muscle layers of the main intestinal segment of the anterior intestine. The serosal saline was acidified after tissue removal but prior to sampling to liberate Cu bound to the incubation vessels. The tissue samples were freeze dried, weighed, and then digested in HNO₃ (68%, Primar-Plus Trace Analysis Grade, Fisher, UK) for 1 h at 60 °C before being diluted and measured for total Cu concentrations (see section 2.4).

The calculation of tissue and rinse metal concentrations and net fluxes across the gut followed Clark *et al.*¹⁷ with modifications for Cu. The concentrations of Cu in the rinses are expressed as percentages (%) of the total doses (ng Cu) injected into the gut sacs at time 0 h. Tissue concentrations of total Cu are expressed as μg g⁻¹ dry weight (dwt) of tissue. The concentrations of Cu in the serosal saline were calculated from the total Cu concentration and volume, then divided by the mass of the muscularis *i.e.* ng mL⁻¹ g⁻¹.

To enable comparison with other studies (*e.g.*, ref. 13 and 21), net Cu uptake rates are also shown expressed as nmol cm⁻² h⁻¹ for 'new' Cu accumulation in mucosa and muscularis (after subtraction of the mean background Cu concentration (nmol Cu per cm) measured in control tissues (as per eqn (2) above but using tissue surface area (SA) rather than tissue dry weight). The SA of the gut tissue was measured by drawing the outline of the muscle layer on paper and the SA calculated using ImageJ software.¹⁷

2.4 Measurements of total copper concentrations

Concentrations of total Cu were measured in samples collected from experiments investigating the dissolution of the CuO ENMs (see section 2.2) and tissues, rinses and serosal saline from the gut sac experiments (see section 2.3) using inductively coupled plasma mass spectrometry, (ICP-MS, Thermo Electron Corporation X-Series II quadrupole). All samples were diluted to volume with an acidified In/Ir solution (internal standard for ICP-MS at a final concentration of 20 μg L⁻¹ In/Ir in 10% HNO₃). Concentrations of Cu were measured and compared to matrix-matched acidified element standards (Fisher, UK). Analytical standards were also measured throughout the run to correct for instrument drift. Procedural blanks (HNO₃ only) were also prepared at the same time as the tissue digests and handled in the same manner as the samples to determine any trace contamination of Cu during sample preparation. The background concentration of Cu in the freshly prepared luminal saline was <0.1 μg L⁻¹, and below the limit of detection (LOD) of the instrument for this matrix.

2.5 Data handling and statistical analyses

All statistical tests were performed with SigmaPlot (v. 13.0, Systat Software Inc.) and data are presented as means ± standard error (SEM) except where stated. All data were tested for normality (Shapiro–Wilk test) and equality of variances (Brown–Forsythe test) and if not normally distributed were

$\log_{10}$ transformed prior to statistical analyses. Statistically significant differences between treatments were detected using student's *t*-tests and one-way ANOVA with Holm-Sidak test *a posteriori*, or where $\log_{10}$ transformation failed to normalise data distributions, Mann-Whitney *U*-tests and Kruskal-Wallis tests with Dunn's multiple comparisons test *a posteriori* were used with untransformed data. A *p* value of <0.05 was considered significant. The data of ENM dissolution over time were fitted with a rectangular hyperbole function.

3. Results

3.1 Behaviour of CuO ENMs under artificial gut conditions

The CuO ENMs were well dispersed in ultrapure water with a median agglomerate size of 110 ± 19 nm, but as expected their introduction into the high ionic strength luminal saline at pH 7.8 led to the formation of larger agglomerates within

2 minutes (Fig. 1A). This agglomeration behaviour quickly lowered the particle number concentration in the dispersion and prevented adequate particle tracking for reliable NTA measurements at later times more relevant to the gut sac exposures *i.e.* after 4 h. For similar reasons, the NTA of ENMs in the saline at different pH *i.e.* pH 2 and pH 4, or in the presence of millimolar concentrations of amino acids was not reliable due to the rapid sedimentation of the materials in the NanoSight viewing chamber.

The dialysis experiments showed that at lower pH of the saline caused an increase in the fraction of total dissolved metal in the external compartment of the beaker (Fig. 1B), which is indicative of dissolution of Cu from the ENMs. At pH 7.8, the fraction of total dissolved Cu released into the beaker was lowest, and became much greater with increasing acidity, *e.g.*, at 4 h (one-way ANOVA, $p < 0.001$, Holm-Sidak tests, $p < 0.001$). Maximum initial dissolution rates of Cu

Q5 Fig. 1 Physicochemical behaviours of CuO ENMs in artificial gut (luminal) saline. Nanoparticle tracking analysis showed CuO ENMs (6.354 mg L^{-1}) formed agglomerates after 2 min in luminal saline at pH 7.8 compared to ultrapure water (A). Dissolution of CuO ENMs was increased in saline at acidic pH (B); 3 mL of an ENM stock at 0.3977 g L^{-1} was added to dialysis tubing and placed in 297 mL saline at pH 2, pH 4 or pH 7.8 and Cu concentrations measured outside of tubing. Analysis of transmission electron micrographs of ENMs incubated for 4 h in saline at pH 4 and pH 7.8 ($n = 100$ ENMs replicate⁻¹, $n = 3$ independent replicates at pH 4 and pH 7.8) showed a shift in the primary particle size distribution of CuO ENMs toward smaller particles at pH 4 (C) and there was a statistically significant decrease in mean particle size (C, inset). At 5 mM, the amino acids L-histidine and L-cysteine also increased dissolution of CuO ENMs at pH 7.8 (D). Data are from 3 independent replicates and where shown are means $\pm$ SEM. Different lower case letters indicate a significant difference between treatment groups (one-way ANOVA or student's *t*-test, $p < 0.05$).

were calculated using the data in the first 25 minutes of the dialysis (*i.e.* where the outward diffusion gradient was maximal) and for 1.1931 mg of Cu loaded into the dialysis tubing at 0 h. The calculated dissolution rates were 1.40 ± 0.10 , 0.08 ± 0.01 and 0.01 ± 0.01 mg h⁻¹ at pH 2, pH 4 and pH 7.8, respectively. In the fixed volume of the beaker, the dissolved Cu fraction after 4 h at pH 2, pH 4 and pH 7.8, was 93.64 ± 2.29 , 64.63 ± 0.44 and $0.65 \pm 0.08\%$ respectively, of the initial added mass of Cu as CuO ENMs. Analysis of the additional controls verified negligible adsorption of dissolved Cu (added as CuSO₄) to the beakers or dialysis tubing in saline at pH 7.8 over 4 h with the measured Cu concentrations at 4 h being $96.70 \pm 0.41\%$ of the initial concentration.

Attempts were also made to determine whether or not the observed apparent dissolution of dissolved Cu from the ENMs caused a decrease in the primary particle size; the latter measured by electron microscopy (Fig. 1C). For saline prepared at pH 4, the CuO ENMs showed a shift in the primary particle diameters toward smaller ENMs compared to the saline at pH 7.8 (Fig. 1C). This was evidenced by a statistically significant difference in the mean primary particle diameter at pH 4 (13.00 ± 0.29 nm) compared to pH 7.8 (18.18 ± 0.33 nm) after 4 h (student's *t*-test, $p < 0.001$; Fig. 1C, inset).

The presence of amino acids in saline (at pH 7.8) also had pronounced effects on the fraction of dissolved Cu released into the external compartment of the beakers during the dialy-

sis experiments (*e.g.*, at 4 h; one-way ANOVA, $p < 0.001$, Holm-Sidak tests, $p \leq 0.01$; Fig. 1D). Incubation with 5 mM L-cysteine or 5 mM L-histidine resulted in 29.11 ± 6.82 and $52.42 \pm 3.65\%$ dissolution respectively, compared to $0.40 \pm 0.03\%$ in saline alone. Maximum initial dissolution rates calculated for the first 25 minutes of the dialysis were 0.04 ± 0.01 and 0.09 ± 0.01 mg h⁻¹ with L-cysteine and L-histidine, respectively.

3.2 Regional differences in uptake of Cu after exposure to CuSO₄ and CuO ENMs in gut sacs

There were clear and consistent differences in the total Cu concentrations in all regions of the gastro-intestinal tract of trout from exposures to CuSO₄ or CuO ENMs respectively (Fig. 2). Approximately 50% of the dosed Cu was recovered in the rinses (Tables 1 and S1†). As expected, there were measureable concentrations of background Cu in control tissues and concentrations were highest in mid and especially posterior regions of the intestine. Exposure to Cu as CuSO₄ led to elevated concentrations of Cu in the mucosal layers of all segments of the intestine. The greatest accumulation of Cu was measured in the mucosa of the mid and posterior regions. The magnitude of increase in concentration of Cu in the mucosa observed with CuSO₄ was also observed in exposures to CuO ENMs. For example, in the mid intestine Cu concentrations in the mucosa were elevated in exposures to

Q6 Fig. 2 Total Cu concentrations in tissue layers in isolated sections of the gastrointestinal tract (A, stomach; B, anterior intestine; C, mid-intestine; D, posterior intestine). See main text for concentrations of Cu in caecae from gut sacs of the anterior intestine. Sections were exposed to 6.3546 mg L⁻¹ Cu (as CuSO₄ or CuO ENMs) in luminal saline for 4 h. Controls were filled with saline, only. Data are means $\pm$ SEM, $n = 5/6$. Data are plotted on same scales to enable comparison between gut regions. Different lower case letters indicate significant differences between treatments within tissue layers (one-way ANOVA, Holm-Sidak test, $p < 0.05$).

Q7 Table 1 Percentage of total Cu (CuSO₄ or CuO ENMs) dosed in gut sacs that was recovered from the luminal saline (rinse 1) and EDTA wash (rinse 2) after 4 h

Treatment	Sample type	Stomach	Anterior intestine	Mid intestine	Posterior intestine
CuSO ₄	Luminal saline	47.3 ± 13.1 ^{a,b}	51.6 ± 8.5 ^a	39.5 ± 5.2 ^{a,c}	39.2 ± 11.5 ^{a,c}
	EDTA wash	19.3 ± 9.4 ^{a,b}	5.3 ± 1.0 ^{a,b}	7.8 ± 0.9 ^{a,b}	7.3 ± 2.3 ^{a,b}
CuO ENMs	Luminal saline	52.1 ± 12.5 ^a	54.3 ± 11.1 ^a	20.5 ± 4.7 ^{a,b}	35.0 ± 4.9 ^{a,b}
	EDTA wash	3.1 ± 1.1 ^b	4.9 ± 3.1 ^{b,c}	4.2 ± 1.4 ^{b,c}	8.4 ± 1.5 ^{a,b}

Data are means ± SEM of $n = 5/6$ samples. Measured Cu concentrations in rinses are shown in Table S1;† background Cu concentrations in controls were subtracted prior to calculations. Values with different lower case letters are significantly different (Kruskal–Wallis test, $p < 0.001$).

both materials compared to controls (one-way ANOVA, F (between-groups degrees of freedom, within-groups degrees of freedom; 2, 13) = 27.352, $p < 0.001$, Holm–Sidak tests $p < 0.001$), but there was no significant difference in concentrations of the two materials (Holm–Sidak test, $p = 0.635$). There was a clear material-type effect on accumulation of the two Cu materials in the muscularis of the gut sacs. This trend was similar across all regions of the gut. For example, measured Cu concentrations in the muscularis of mid intestines were significantly different between treatments (one-way ANOVA, $F(2, 14) = 13.071$, $p < 0.001$) with Holm–Sidak *post hoc* analyses indicating CuSO₄ treated intestines were elevated compared to controls ($p = 0.001$) and CuO ENMs ($p = 0.002$), but there were no significant difference between CuO ENMs and controls ($p = 0.924$). Measured concentrations in caecae of gut sacs of the anterior intestine region were not significantly different between treatments and are not shown in Fig. 2 but were 2.19 ± 1.14 , 3.81 ± 1.52 and $2.57 \pm 0.26 \mu\text{g g}^{-1}$ in gut sacs exposed to control, CuSO₄ or CuO ENMs, respectively ($p = 0.093$).

Based on these uptake rates into the muscularis (likely into the blood space), the combined total dietary Cu uptake per unit mass of the trout used in these experiments and exposed to $100 \mu\text{mol L}^{-1}$ Cu (as CuSO₄) would equate to $0.116 \pm 0.04 \text{ nmol g}^{-1}$ trout across the gastrointestinal tract over the 4 h period of the experiment. A broadly similar trend to that observed in the muscularis (Fig. 2) was also apparent for Cu concentrations in serosal salines although the high variability between replicates, including in the control group, did not lend themselves to useful comparisons (Table 2). Overall, these data indicated that the highest rates of uptake/association of dissolved Cu were in the mucosa of the mid and pos-

terior intestines, and with comparable uptake of dissolved Cu uptake into the muscularis in all tissues.

Net fluid fluxes in the tissues were not significantly different between treatment groups (Kruskal–Wallis test, $p > 0.05$; Table 2). Broadly speaking, there were no consistent trends in the data to demonstrate a net loss or gain of fluid in the stomach, anterior and posterior regions of the gut over the 4 h flux period. In the mid-intestine, there was a clear net gain of fluid from the gut sacs that was apparent in all treatment groups (Tables 2 and S6 and S7†).

The mean percentage of ‘new’ Cu accumulation within each tissue compartment (caecae, mucosa, muscularis or serosal saline) are shown in Table 3. This represents the proportion of newly acquired Cu in each tissue compartment after deducting the background Cu concentration measured in the unexposed controls. The trends in the data show that within the tissue compartments the Cu dose was largely associated with the mucosa and only a few percent was found in the muscularis or serosal saline. There were few statistically significant differences between gut regions or treatments.

3.3 Effects of amino acids on uptake of CuO ENMs in gut sacs

The concentrations of Cu in the rinses are shown in Tables S2–S5.† L-Histidine had little effect on the accumulation of Cu in the tissues for exposures to either CuSO₄ or CuO ENMs into the mucosa or muscularis and in both the mid and posterior regions of the gastrointestinal tract (Fig. 3). One-way ANOVAs indicated that concentrations of Cu measured in the mucosal layer of the mid [$F(4, 24) = 13.423$, $p < 0.001$] and posterior intestine [$F(4, 24) = 7.824$, $p < 0.001$] were significantly elevated for all material and amino acid combinations

Q8 Table 2 Fluid flux and total Cu accumulation into the serosal compartment of gut sacs

Treatment	Stomach	Anterior intestine	Mid intestine	Posterior intestine
Total Cu concentration in serosal saline ($\mu\text{g mL}^{-1} \text{ g}^{-1}$)				
Control	0.03 ± 0.01 ^{a,b,c}	0.21 ± 0.01 ^{a,b,c}	0.64 ± 0.17 ^{a,b,c}	0.70 ± 0.13 ^{a,b,c}
CuSO ₄	0.02 ± 0.01 ^{a,b}	0.59 ± 0.19 ^{a,b,c}	1.07 ± 0.31 ^{b,c}	1.49 ± 0.57 ^c
CuO ENMs	0.01 ± 0.01 ^a	0.32 ± 0.06 ^{a,b,c}	0.59 ± 0.10 ^{a,b,c}	0.81 ± 0.22 ^{b,c}
Fluid flux ($\mu\text{L g}^{-1} \text{ h}^{-1}$)				
Control	−4.3 ± 0.4 ^{a,b}	2.6 ± 3.3 ^{a,b}	52.1 ± 9.6 ^{a,b}	35.1 ± 25.8 ^{a,b}
CuSO ₄	0.4 ± 5.8 ^{a,b}	13.1 ± 4.8 ^{a,b}	70.6 ± 17.0 ^b	20.0 ± 12.4 ^{a,b}
CuO ENMs	−2.5 ± 6.9 ^a	6.0 ± 6.1 ^{a,b}	53.1 ± 4.4 ^{a,b}	0.3 ± 5.5 ^{a,b}

Data are means ± SEM of $n = 5/6$ samples. Different lowercase letters indicate significant differences between treatments (Kruskal–Wallis tests, $p < 0.05$).

Table 3 Partitioning of Cu throughout the gut sac tissue compartments expressed as a percentage of the Cu dose at the start of the 4 h incubation

Treatment	Region of gut	Caecae	Mucosa	Muscularis	Serosal saline
CuSO ₄	Stomach		4.5 ± 2.0 ^{a,b,c,d}	1.8 ± 0.4 ^{a,b,c,d}	0.1 ± 0.1 ^a
	Anterior intestine	24.4 ± 8.8 ^{c,d}	4.4 ± 2.2 ^{a,b,c,d}	3.3 ± 0.8 ^{a,b,c,d}	0.2 ± 0.1 ^{a,b,c,d}
	Mid intestine		12.1 ± 2.0 ^{c,d}	3.8 ± 1.4 ^{a,b,c,d}	0.4 ± 0.2 ^{a,b,c,d}
	Posterior intestine		12.1 ± 4.0 ^{b,c,d}	4.3 ± 1.0 ^{a,b,c,d}	0.5 ± 0.2 ^{a,b,c,d}
CuO ENMs	Stomach		1.2 ± 0.3 ^{a,b,c,d}	0.4 ± 0.2 ^{a,b,c,d}	0 ± 0 ^a
	Anterior intestine	6.9 ± 3.0 ^{a,b,c,d}	2.6 ± 0.5 ^{a,b,c,d}	0.3 ± 0.2 ^{a,b,c,d}	0.1 ± 0.1 ^{a,b,c}
	Mid intestine		14.4 ± 3.1 ^d	0.1 ± 0.1 ^{a,b}	0.1 ± 0.1 ^{a,b}
	Posterior intestine		15.2 ± 4.4 ^{c,d}	1.4 ± 0.2 ^{a,b,c,d}	0.1 ± 0.1 ^{a,b}

Data are means ± SEM of $n = 5/6$ samples. Background Cu concentrations in controls are shown in Fig. 2 and were subtracted prior to calculations. Values with different lower case letters are significantly different (Kruskal–Wallis test, $p < 0.001$).

compared to controls, but there were otherwise no differences between the treatments. The accumulation of Cu from CuSO₄ and CuO ENMs into the muscularis were unaffected by L-histidine in both mid and posterior intestine and the same patterns of accumulation were evident in both tissues. Greater concentrations of Cu were observed in muscle layers of gut sacs filled with CuSO₄ compared to both CuO ENMs and controls, irrespective of co-exposure with L-histidine. The appearance of total Cu in the serosal compartment was

small, and considering variations in the tissue mass, there was not sufficient resolution to determine an effect of this amino acid on apparent net uptake to the serosal compartment (Tables S6 and S8†).

The effect of additions of L-cysteine were also explored (Fig. 3), but in general additions of L-cysteine had little effect on Cu accumulation in the tissue from the CuSO₄ exposure. In contrast to L-histidine, L-cysteine significantly altered the accumulation of Cu in the tissues from CuO ENM exposures

Fig. 3 Total Cu concentrations in tissue layers in isolated sections of the mid (A and C) and posterior (B and D) intestines. Sections were exposed to 6.3546 mg L⁻¹ Cu (as CuSO₄ or CuO ENMs) and with or without 5 mM L-histidine (A and B) or 5 mM L-cysteine (C and D) in luminal saline for 4 h. Controls were filled with saline, only. Data are means ± SEM, $n = 5/6$. Different lower case letters indicate significant differences between treatments within tissue layers (one-way ANOVA, Holm–Sidak test, $p < 0.05$).

(Fig. 3). In the mid intestine, the accumulation of Cu in the mucosa from exposure to CuO ENMs was significantly greater in the presence of L-cysteine [one-way ANOVA, $F(4, 18) = 18.782$, $p < 0.001$, Holm–Sidak test $p < 0.001$]. The effect of L-cysteine on accumulation of Cu from CuO ENMs in the muscularis was even more pronounced. One-way ANOVA [$F(4, 18) = 26.649$, $p < 0.001$] indicated significantly greater Cu concentrations in muscularis of mid intestines filled with CuO ENMs and L-cysteine compared to CuO ENMs alone (Holm–Sidak test $p < 0.001$) or controls (Holm–Sidak test $p < 0.001$). A similar pattern of elevated Cu accumulation from CuO ENM exposure in the presence of L-cysteine was also evident in the posterior intestine. Accumulation of Cu in the serosal saline was negligible (Tables S7 and S9†).

4. Discussion

The present study shows that the net metal accumulation from an ENM exposure can be measured in a few hours using the *ex vivo* gut sac of fish. It also informed on the relative hazard with the tissue total Cu concentrations from exposure to CuO ENMs being similar to, or much less than, those of the equivalent metal salt, CuSO₄. The agglomeration behaviour of CuO ENMs in the high ionic strength of the luminal saline would likely cause particle settling on the gut epithelium and therefore ensure exposure. The CuO ENMs showed greater dissolution at low pH and in the presence of amino acids. Crucially, dissolution and subsequent Cu complexation with amino acids suggest a bioavailable sink of Cu metal derived from the ENMs. In the case of added cysteine, this led to greater total Cu accumulation in intestinal tissues.

4.1 Copper bioavailability and accumulation in the presence of luminal saline

A key determinant in the uptake of metals and metallic ENMs at the intestine in fish is the form and concentration of the material presented at the mucous epithelium.²³ The speciation of Cu from CuSO₄ in the saline was calculated using Visual MINTEQ 3.1 (ref. 37) and these data indicated that Cu would be predominantly complexed with carbonate (68.15% CuCO₃ (aq) and 29.38% Cu(CO₃)₂²⁻) with only 0.71% as Cu²⁺. The high ionic strength luminal saline used promoted the formation of agglomerates of the ENM within a few minutes at the normal pH of 7.8. Indeed, the agglomeration was so effective that insufficient particles remained in dispersion after 4 h in the saline, such that NTA measurements became unreliable (*i.e.*, not enough agglomerates to track). This finding is consistent with some of our previous studies on other ENMs in the same luminal saline^{16,17} where particle settling onto the tissue was also observed. In the gut sacs, the tendency of the luminal saline to cause rapid particle settling would therefore help present the ENMs to the exterior surface of the mucosa.

The multiple rinses, which also included an EDTA rinse, enabled the surface adsorbed fraction to be removed so that the total Cu accumulated firmly on or in the tissue could be

measured. Gut sacs from the unexposed control animals showed a background Cu concentration of around 2–10 µg g⁻¹ dwt, depending on the tissue and tissue compartment (Fig. 2), and this is broadly similar to rainbow trout *in vivo* (6 µg g⁻¹ dwt for the whole gut).³⁸ The gut sacs exposed to CuSO₄ for 4 h at pH 7.8 showed elevated total Cu concentrations in the tissues compared to the controls and with around two thirds or more of the Cu in/on the mucosa rather than the muscularis (Fig. 2); and entirely in keeping with previous findings on dissolved Cu in gut sacs (data of Cu uptake normalised to tissue area are shown in Table S10†).^{12,13,21} The Cu from CuSO₄ exposure was also accumulated more in the mid- and posterior-intestine than the stomach or anterior intestine (Fig. 2), as expected from previous reports.¹² In exposures with the CuO ENMs, the relative accumulation of total Cu in the mucosa was higher than the muscularis, and the overall pattern of Cu accumulation in the regions of the gut were similar to that observed with CuSO₄ (Fig. 2); with two exceptions. The stomach mucosa accumulated slightly less Cu during exposures to CuO ENMs compared to CuSO₄ (Fig. 2), but more important, unlike the exposures CuSO₄, there were no increases in Cu concentrations in the muscularis with the CuO ENMs. This suggests that the CuO ENMs are retained in the mucus in particulate form and less Cu from CuO ENMs is passing through the mucosa into the underlying tissues and blood space leading to the serosal compartment.

From a hazard perspective, any Cu that can pass through the mucosa into the underlying tissue and serosal compartment would represent an exposure risk to other internal organs of the animal *in vivo*. The gut sac preparation here is a ‘closed system’ of a finite volume, and is not intended to provide values for the maximum possible transepithelial Cu flux (unlike the perfused intestine, where the serosal fluid is constantly replaced¹²). Nonetheless, the appearance of additional Cu (above that of the unexposed control) in the serosal compartment would be indicative that net uptake of Cu to the blood side is possible *in vivo*. A small fraction of the luminal dose of Cu from CuSO₄ was detected in the serosal fluid (Table 2). This is consistent with the notion that transepithelial Cu uptake also occurs *in vivo*^{19,38} and is nutritionally essential.¹⁸ In contrast, Cu concentrations in serosal salines of gut sacs filled with CuO ENMs did not increase above control levels. This suggests that while the ENMs provided Cu that was bioavailable to the mucosa, it was not quickly transported (form unknown) into the blood side of the gut sacs and would therefore not be bioavailable to the internal organs *in vivo* within a few hours of exposure. However, the aetiology of organ pathologies for Cu ENM exposures is slower to appear than those from the metal salt *in vivo*³⁹ and it may simply be that the uptake of CuO ENMs is slower because it uses a different pathway to the metal salt. ENMs are likely too large for solute transporters,²³ such as those used for Cu ions (Ctr1 and Cu-ATPases⁴⁰), and any intact particles would be taken up by slower endocytosis pathways as observed for Caco-2 cells.⁴¹ It is also likely that strands of mucus on the gut surface would impair the diffusion of particles to the apical membrane.²³ Nevertheless, from the view point of

total Cu accumulation in the muscularis and the serosal saline at normal pH and although interactions between CuO ENMs and food particles have not been fully explored in the present study (but see discussion on interactions with amino acids below), the bioaccumulation hazard to the gut for CuO ENMs is less than that of the metal salt. Therefore, the existing dietary exposure risk assessment for Cu in fish,⁴² might also be protective for the nanoform.

4.2 Effects of pH and amino acids on transformation and bioavailability of CuO ENMs to the gut

The acidity in the stomach of trout can range between pH 2 and pH 4 during digestion,³³ and the effect of such low pH values on ENM behaviour in the luminal saline was explored (Fig. 1). Similar to luminal saline at pH 7.8, agglomeration of the CuO ENMs was near instantaneous. However, unlike conditions at pH 7.8, the high $[H^+]$ at low pH led to progressively greater dissolution of Cu^{2+} , and at pH 2 more than 90% of the Cu from CuO ENMs became dissolved. This effect of low pH on the dissolution of Cu ENMs has been previously observed.^{29,43} It also resulted in smaller particles of the CuO ENMs (Fig. 1), and so after digestion at low pH in the stomach, the nanomaterial will be presented to the gut mostly as dissolved Cu, but also with some remaining in the particulate form over 4 h, depending on the level of acidity. The bioaccumulation potential of smaller acid-treated CuO ENMs was not explored in the present study.

Acid digestion of food in the stomach would be expected to release Cu binding ligands from food, *e.g.*, amino acids. Notably, amino acids promoted dissolution of the CuO ENMs in saline at pH 7.8 (Fig. 1D), and likely because $-NH$ and $-SH$ ligands in histidine and cysteine, respectively⁴⁴ acted as Cu^{2+} ion chelators (*i.e.*, sinks for binding Cu^{2+} ions so that dissolution of Cu from surface of the ENMs was promoted). There was also a clear difference in the magnitude of effect of the amino acids; dissolution of CuO ENMs was two-fold greater with L-histidine compared to L-cysteine. This stability constant for Cu^{2+} complexation with histidine and cysteine are similar ($\log\beta$, 17.50 and 17.98 respectively).⁴⁵ However, once the complex is formed, in the case of Cu-cysteine, it is essentially irreversible (dissociation constant 6.5×10^{20}),³⁰ but much less so for the amino group in histidine ($\sim 1.6 \times 10^9$).⁴⁶ So for example, once the cysteine is used up, dissolution may slow or even stop. Regardless, this phenomenon has been documented previously where the dissolution of CuO ENMs was much greater with histidine than cysteine.⁴³

Notably, enhanced dissolution of the CuO ENMs in the presence of L-histidine did not correspond with increased total Cu accumulation in gut compartments (Fig. 3). There was no difference in the total Cu accumulation in mucosa with or without L-histidine in the presence of CuO ENMs, and with L-histidine, the muscularis showed a decrease in total Cu (Fig. 3). This suggests that any Cu-histidine complexes formed *via* particle dissolution did not have great bioavailability, or might even slow Cu accumulation. This is contrary to

some previous reports in rainbow trout with dissolved Cu. For example, L-histidine has been shown to increase copper transport in gut sac and brush border membrane preparations.^{13,22} In the present study, Cu accumulation in the presence of $CuSO_4$ exposure with L-histidine was also normal with no evidence of inhibition of Cu accumulation in the muscularis, unlike the situation with CuO ENMs (Fig. 3). For metal salt exposures, the Cu-histidine complex is normally present *in vivo* and bioavailable. The Cu-histidine complex in the blood compartment, for example, is delivered and uncoupled at cell membrane, where histidine stimulated NADH oxidase provides an electron for the uptake of Cu as Cu^+ .⁴⁷ Interestingly, nanoparticles are known to inhibit NADH oxidase activity (*e.g.*, Au NPs⁴⁸), and this may explain the difference in response to $CuSO_4$ and CuO ENMs in the muscularis.

A further possible reason for negligible increases in Cu accumulation in the gut sacs with L-histidine is that Cu uptake is also speciation-dependent,²² and the speciation effect may mask any benefit of L-histidine additions. The high $[HCO_3^-]$ used in the present study, but not in the physiological saline used by Nadella *et al.*¹³ and Glover *et al.*²² has also been shown to decrease the rate of Cu uptake in Caco-2 cells.⁴⁹ The putative Cu-Cl symport in fish intestine would also slow with HCO_3^- as a competing ion for chloride.¹² Unfortunately, chemical speciation models are not yet available for salines containing ENMs.

In contrast, L-cysteine increased the accumulation of total Cu in gut sacs from CuO ENM exposures, including in the muscularis. L-Cysteine similarly elevated the accumulation of $CuSO_4$. These data suggest that either, L-cysteine facilitated delivery of Cu to dissolved Cu transport proteins at the apical membrane of gut (*e.g.*, Ctr1 and/or Dmt1), or a distinct pathway transports the Cu-cysteine chelate into the cell, as has been previously proposed for histidine.²² This latter scenario would, if the pathway were amino acid-specific,⁵⁰ explain the discrepancy between the effects of L-cysteine and L-histidine on Cu transport in gut sacs. Alternatively, in excess, Cu can catalyse the autoxidation of L-cysteine with the subsequent reduction of Cu(II) to Cu(I).⁵¹ The latter would be available as the main substrate for Ctr1 at the apical membrane.

4.3 Conclusions and including physiological factors in the regulatory testing of engineered nanomaterials

In conclusion, this study has demonstrated the utility of the gut sac preparation for an ENM made of an essential metal, copper, which is nutritionally required by fish. In contrast to our previous gut sac studies on non-essential metals,^{16,17} there is an endogenous pathway for the physiological uptake of dissolved Cu in the gut that also has a vesicular component.¹² Under the normally acidic conditions found in the stomach, the CuO ENMs show substantial dissolution, and the subsequent bioavailability of any dissolved Cu released to the intestine will be modulated by known physiological processes such as likely chelation with amino acids in the gut lumen and amino-acid dependent Cu-uptake. For regulatory use, the gut sac method is intended to be standardised and

using the luminal saline.⁴ For nutritive metals found in ENMs, it will not be practical to test every possible modulating factor. Instead, a decision tree should be developed that informs on whether or not to proceed to *in vivo* testing. For the gut sac, a key trigger could be the appearance of the test substance in muscularis or serosal compartment, which would imply potential uptake to the internal organs *in vivo*. Dissolution rates and known speciation chemistry in the gut saline could provide factors for any additional hazard of dissolved metal fractions from the particles. While the transformation of the ENMs in the gut lumen may be important. One should also not forget that the gastrointestinal tract is an organ system that is vital for survival. Failure of net fluid flux or the integrity of the preparation would also inform any acute toxic hazard *via* the oral route. Overall, for CuO ENMs at least, provided the effect of cysteine especially is considered, the bioaccumulation for total Cu in the gut is broadly similar to that of CuSO₄ exposure.

Conflicts of interest

There are no conflicts to declare.

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